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COMPARATIVE EVALUATION OF QUANTITATIVE TEST METHODS FOR GASES ON A HARD SURFACE

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14. ABSTRACT:
In this study, two quantitative test methods were evaluated (the Three Step Method [TSM] and the Quantitative Disk Carrier Test [QCT]) for determining the efficacy of chlorine dioxide (CD) gas and vaporous hydrogen peroxide (VHP) against spores of *Bacillus subtilis*. The tests were performed at three efficacy levels (low, medium, and high). Steel carriers, inoculated with 7 logs of spores, were exposed to 250, 500, and 3000 parts per million volume (ppmv/h) concentration × time (CT) of CD gas or 150, 450, and 900 ppmv/h CT of VHP. For the high-CD treatment, testing resulted in a narrow range of log reduction (LR; 6–7) for both methods; however, for the medium- and low-CD treatments, the variable LR values ranged between 4 and 5 for QCT and 2 and 4.5 for TSM. The standard deviation (SD; 0.5–2.5 log) was high for the medium- and low-CD treatments. For VHP, a value of >6 LR was observed with QCT for each treatment level, and mean LR values of 2, 3.5, and 4.5 for the low, medium, and high treatment levels, respectively, were observed with TSM. TSM was more sensitive to the treatment level, and the high variability could have resulted from poor gas penetration.

15. SUBJECT TERMS
Three Step Method (TSM) | Quantitative Carrier Disk Test (QCT) | Chlorine dioxide (CD)
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PREFACE

The work described in this report was authorized under U.S. Environmental Protection Agency, Office of Pesticide Programs contract no. IAG DW-21-83021801-2. The work was started in April 2008 and completed in December 2008.

The use of either trade or manufacturers' names in this report does not constitute an official endorsement of any commercial products. This report may not be cited for purposes of advertisement.

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COMPARATIVE EVALUATION OF QUANTITATIVE TEST METHODS FOR GASES ON A HARD SURFACE

1. INTRODUCTION

Members of the U.S. Environmental Protection Agency, Office of Pesticide Programs (USEPA OPP) were interested in selecting a quantitative test method for evaluating the efficacy of two gaseous sterilants: chlorine dioxide (CD) gas and vaporous hydrogen peroxide (VHP). They were unable to locate any validated laboratory methods for testing gases. Validated methods are crucial to the development and potential registration of antimicrobial products that are designed for the decontamination of environmental surfaces, facilities, personal protective gear, and equipment in the event of a biological weapons attack involving spores of *Bacillus anthracis* (Rastogi et al., 2013).

The USEPA OPP members selected the following validated methods used for liquid disinfectants (Springthorpe and Sattar, 2007; Perez et al., 2005):

- the Three Step Method (TSM), a modified form of the AOAC Method 2008-05 (AOAC, International; Rockville, MD) and
- the Quantitative Carrier Disk Test (QCT), a modified form of the ASTM Method E2197-02 (ASTM International; West Conshohocken, PA).

The AOAC Method 2008-05 (TSM) uses three coupons (5 × 5 × 1 mm each) for each chemical treatment to deliver spores into 400 µL of liquid sporicidal agent contained in a micro-centrifuge tube. After exposure to the test chemical and associated neutralization, spores are removed from the carriers in three fractions (A, B, and C) by loosely washing (fraction A), sonicating (fraction B), and gently agitating (fraction C). Liquid from each fraction is placed onto recovery medium to determine the log reduction (LR; i.e., the number of viable spores). Control carrier populations are compared to the treated counts, and the level of efficacy is determined by calculating the LR of each sample (Rastogi et al., 2013).

The ASTM Method E2197-02 (QCT) uses a brushed stainless steel disc as the coupon, which is placed in a glass vial, vortexed after neutralization at the appropriate time, diluted and filtered, and then placed on the appropriate media plates. The LR for both methods is calculated in the same manner.

In this study, the tests were performed at three efficacy levels (low, medium, and high), and the coupons of both methods were compared side-by-side after they had been inoculated with *B. anthracis* and fumigated with either VHP or CD gas.

2. MATERIALS AND METHODS

2.1 Strain Information, Coupon Inoculation, and Storage

The *Bacillus subtilis* spores were generated in the same manner as those of the AOAC Method 2008-05 on nutrient agar amended with manganese (Tomasino et al., 2008). A working stock with a titer of 1×10^9 spores/mL was stored. No bioburden was added to the spore preparation. A 10 µL aliquot was inoculated onto each test coupon (stainless steel for the AOAC Method 2008-05 and brushed steel for the ASTM Method E2197-02), in accordance with the protocol. The acceptable target spore density per coupon was 1.0×10^7, with the acceptable range being between 5.0×10^6 and 5.0×10^7 spores/coupon. The coupons were dried in a biosafety cabinet (BSC) in an open petri dish for a minimum of 1 h and then placed in a Fisherbrand glass desiccator (Pittsburgh, PA) at room temperature for a minimum of 12 ± 2 h before use. Visible dryness of the coupons was observed between 1 and 2 h. The inoculated coupons could be stored for up to 30 days under desiccation conditions.

2.2 Coupon Cleaning and Sterilization

Steel carriers were used for both methods. The steel carriers were cleaned by three alternate washes with deionized water and 70% ethanol and then dried overnight at room temperature. The next morning, the carriers were autoclaved (Steris Corporation, Inc.; Mentor, OH) on a dry cycle (45 min of sterilization and 30 min of drying). Sterility checks were done by placing 2% of the autoclaved carriers into 1 mL of tryptic soy broth in an Eppendorf tube and then incubating for 5 days at 37 °C. The absence of turbidity indicated that the carriers had passed the sterility check.

2.3 Fumigants

The two fumigants used in this research were VHP (Steris Corporation) and CD gas (ClorDiSys, Inc.; Lebanon, NJ). The VHP was generated using the Steris M100 generator, and the carriers were placed in a D box that was hooked up to the generator (Figure 1). CD was generated using the ClorDiSys generator (Figure 2). The target CT (concentration × time) for VHP was 150 ppmv/h for the low treatment, 450 parts per million volume (ppmv/h) for the medium treatment, and 900 ppmv/h for the high treatment. The VHP concentration was measured every 5 min with a Drager sensor (Drager, Inc.; Telford, PA), and the standard deviation (SD) from the mean was <15%. The target CT for CD was 250 ppmv/h for the low treatment, 500 ppmv/h for the medium treatment, and 3000 ppmv/h for the high treatment. The CD gas was measured every minute spectrophotometrically, and these results were confirmed by the titration method. The SD of the CD concentration in parts per million volume from the mean was <10%.

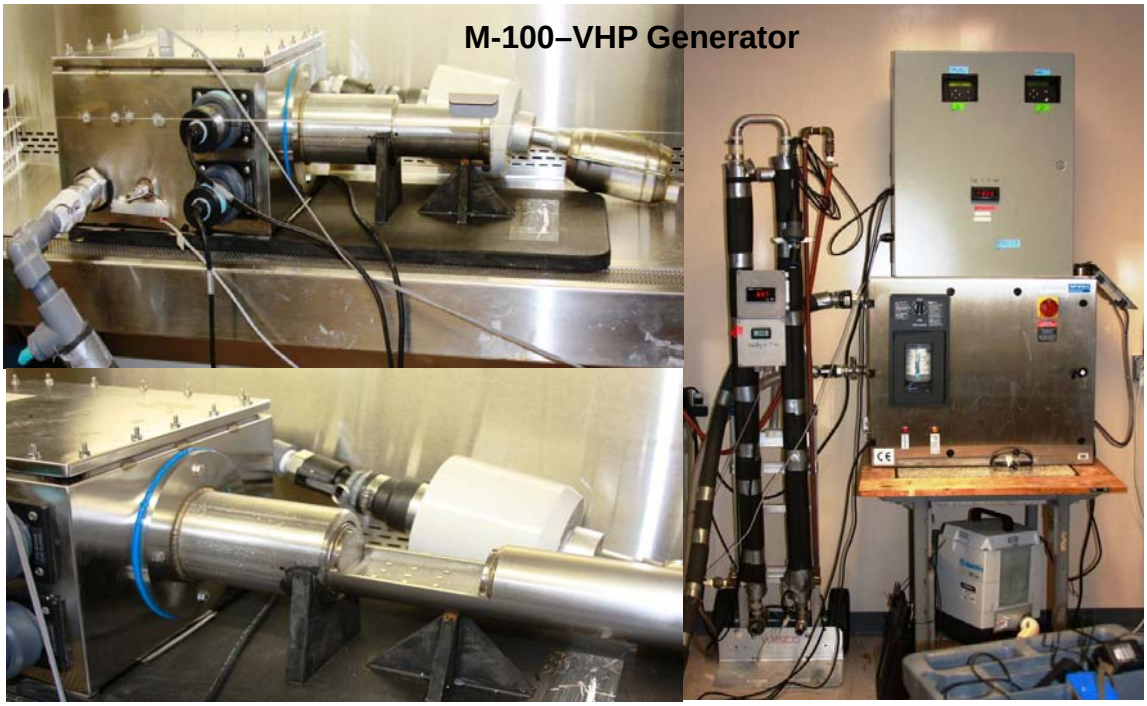

Figure 1. M100 VHP generator and D box.

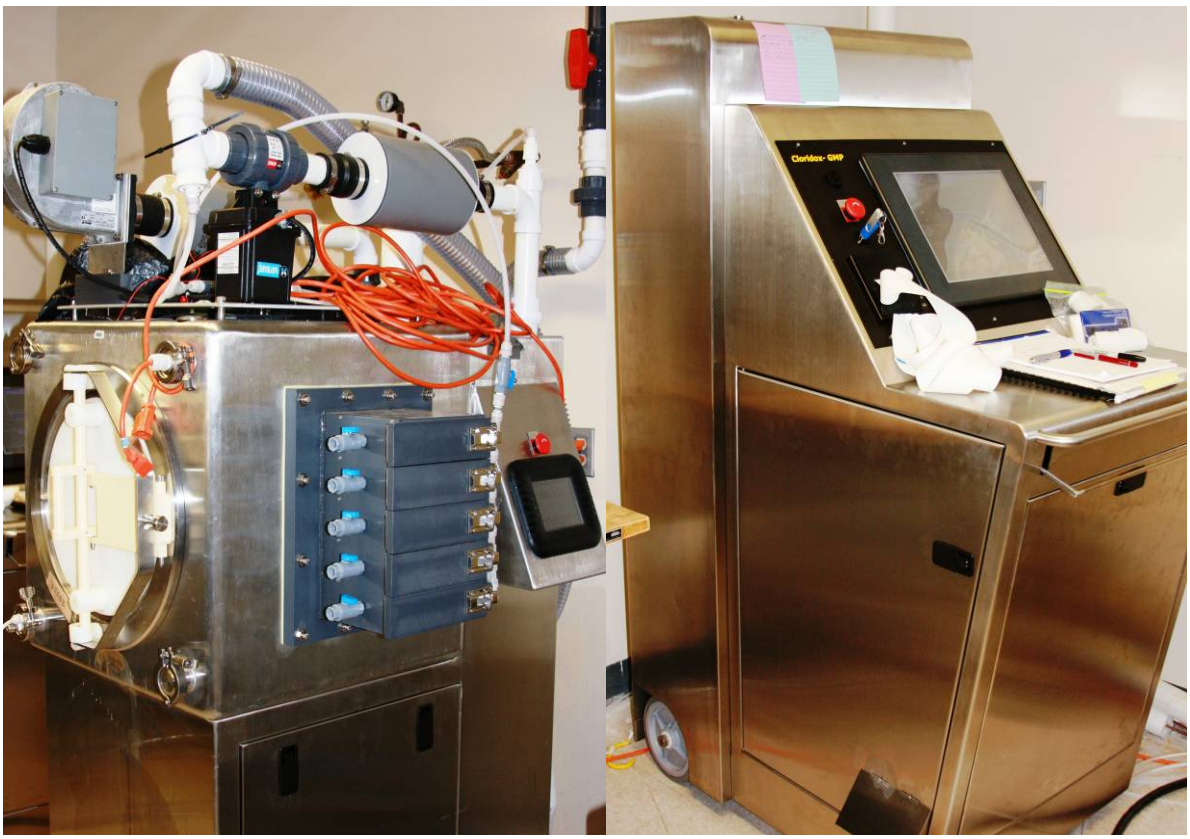

Figure 2. CD chamber and generator.

2.4 Experimental Design

Each quantitative method was performed three times on three consecutive days. For the CD runs, three petri plates, each containing three inoculated carriers, were placed in the chamber. In addition, three carriers in another plate (not fumigated) served as the positive controls. Each of the three petri plates in the chamber were withdrawn after an appropriate exposure period. For the VHP runs, nine carriers were placed in the D-tube, and a set of three carriers were withdrawn after a specified exposure period. For neutralization of the active ingredient, all test carriers were aerated in a biosafety level (BSL)-2 cabinet for 4 ± 1 min, before they were transferred to either a 1.5 mL Eppendorf tube (AOAC Method 2008-05) or a glass vial (ASTM Method E2197-02). No chemical neutralizer was used. The carriers were then processed for spore extraction in accordance with the respective protocols of the two methods. For the test design (Table 1), only one fumigant was evaluated at a time. The exposure times for CD were 15, 30, and 180 min using 1000 ppmv of gas. The exposure times for VHP were 30, 90, and 180 min using 300 ppmv of the fumigant.

Table 1. Test Design

Repeat No.	Expected Efficacy Level*	CD or VHP	
		QCT-2	TSM
CD repeat 1	High, Medium, low, Water Control	August 25, 2008	July 9, 2008
VHP repeat 1		August 6, 2008	June 25, 2008
CD repeat 2		August 27, 2008	July 23, 2008
VHP repeat 2		August 11, 2008	June 30, 2008
CD repeat 3		September 9, 2008	July 28, 2008
VHP repeat 3		August 13, 2008	July 21, 2008

*Three runs were performed for each treatment level.

3. RESULTS

3.1 Fumigant Concentrations

The VHP concentration for all of the experimental runs was 300 ± 50 ppm (Figure 3). The CD concentration for all of the experimental runs was 2.8 ± 2 mg/L.

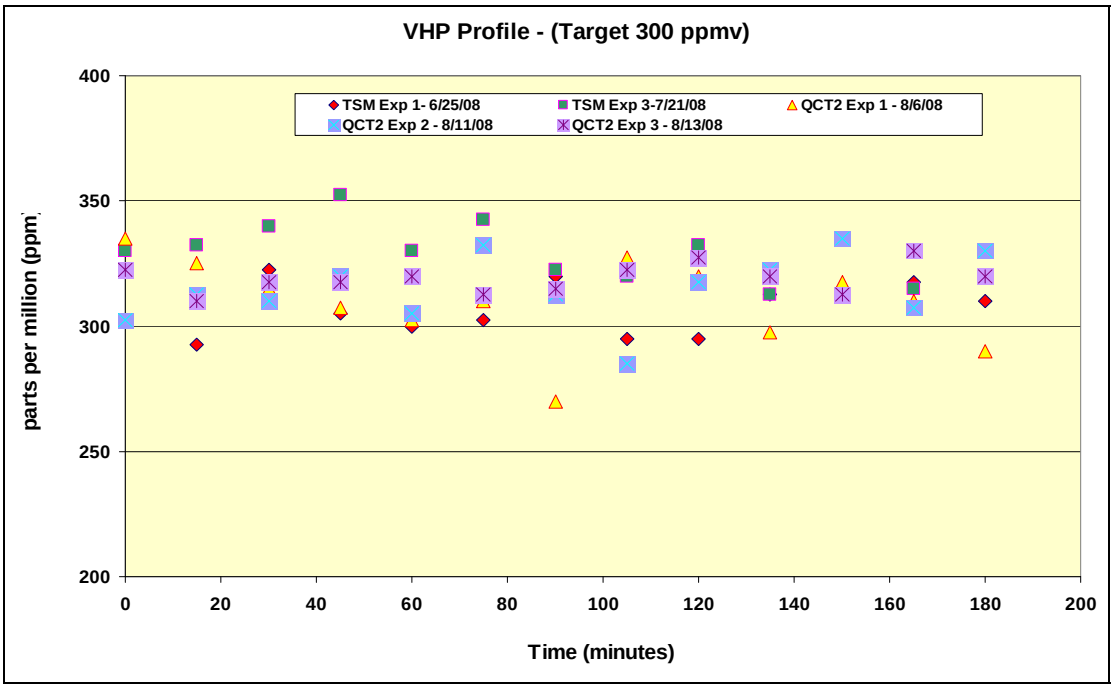

Figure 3. VHP concentration for all experimental repeats.

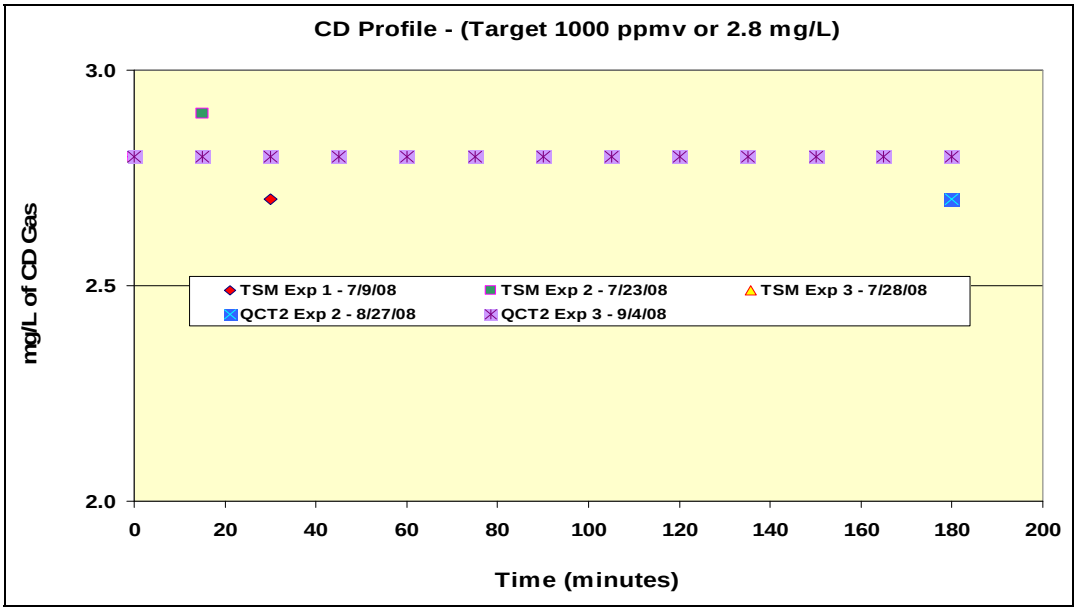

Figure 4. CD concentration for all experimental repeats.

3.2 Spore Recovery of Control Carriers

The percent spore recovery for each coupon type was calculated using the following equation:

[Total recovered colony-forming units (i.e., fractions A + B + C) per number of spores in 10 mL aliquot spotted] × 100

Example: $[2.7 \times 10^7/3.4 \times 10^7] \times 100 = 83\%$

No huge difference was observed in the percent spore recoveries of the two methods. Both results showed between 60 and 80% of the *B. subtilis* amount that had been inoculated on the steel carriers (Figure 5). Comparison of the log colony-forming units of the mean spore recovery showed no difference between the two methods (Figure 6).

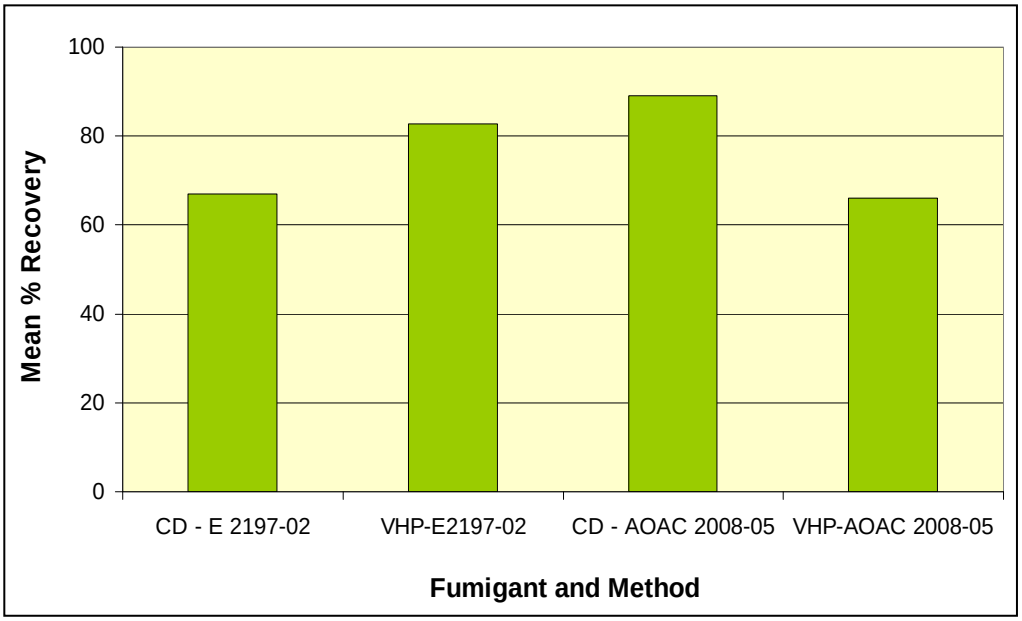

Figure 5. Percent spore recovery of both methods.

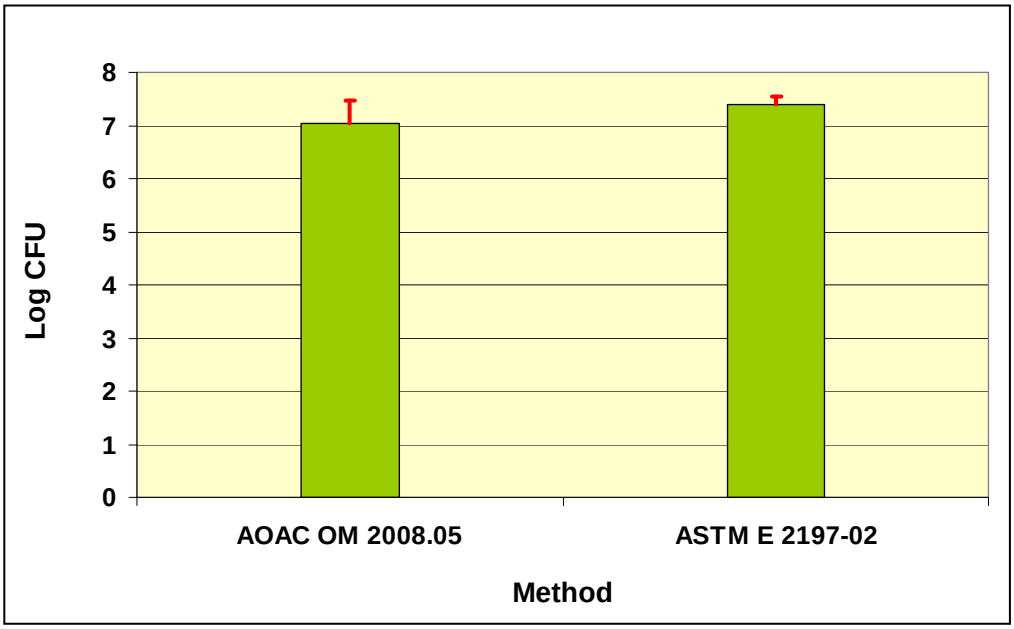

Figure 6. Comparison of mean spore recovery for both methods.

3.3 Method Performance CD Gas Efficacy

The mean LR values from a set of three replicate carriers using AOAC Method 2008-05 are shown in Figure 7. The error bars illustrate the SD in parts per million volume per hour after exposure to CT values.

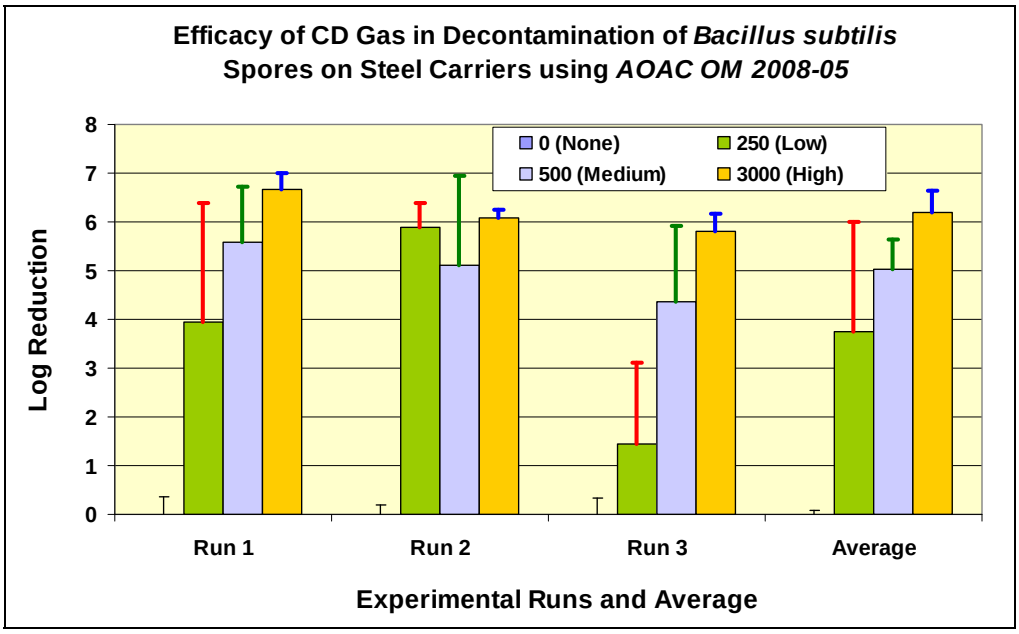

Figure 7. CD decontamination efficacy of *B. subtilis* spores on steel carriers using AOAC Method OM 2008-05.

The mean LR values from a set of three replicate carriers using ASTM Method E2197-02 are shown in Figure 8. The error bars show the SD after exposure to CT values in parts per million volume per hour. The method performance of CD gas is summarized in Figure 9. Figure 12 and Table 2 summarize the performance comparison of the two methods.

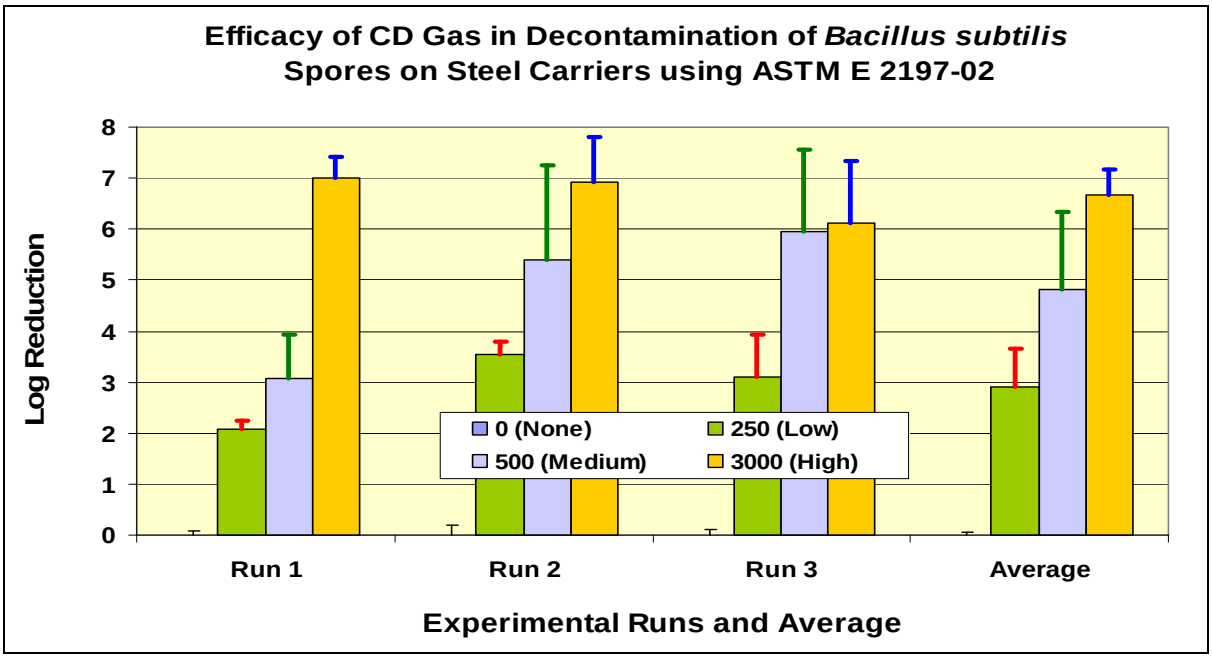

Figure 8. CD decontamination efficacy of *B. subtilis* spores on steel carriers using ASTM Method E2197-02.

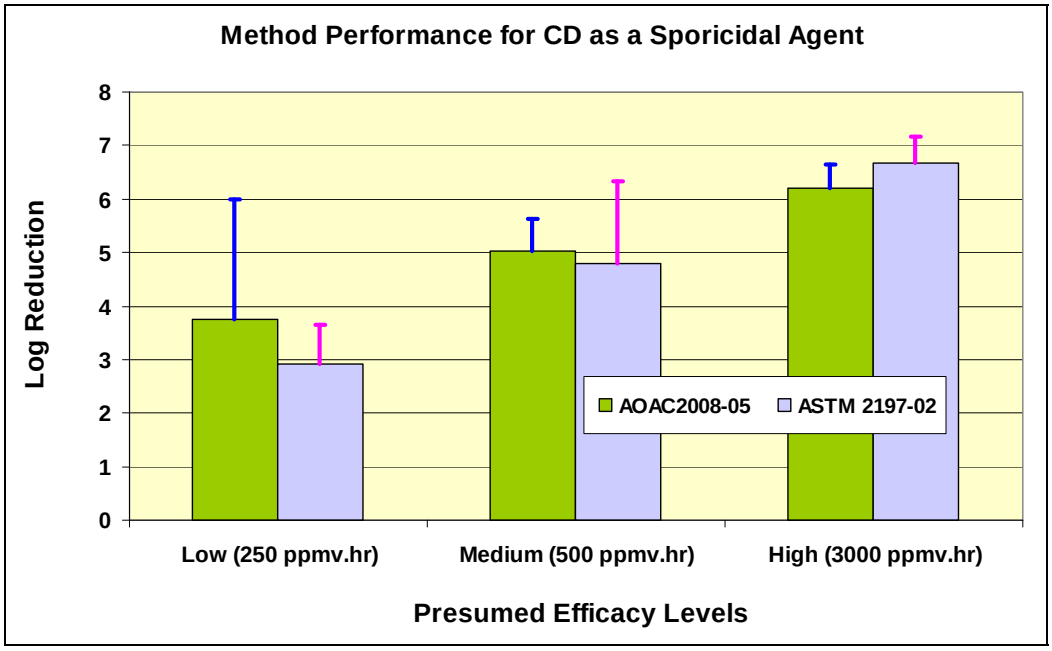

Figure 9. Side-by-side comparison of LR values of each method after CD gas fumigation.

3.4 Method Performance of VHP Efficacy

The efficacy and performance of VHP data are presented in Figures 10 and 11. Figure 12 and Table 2 summarize the performance comparison of the two methods.

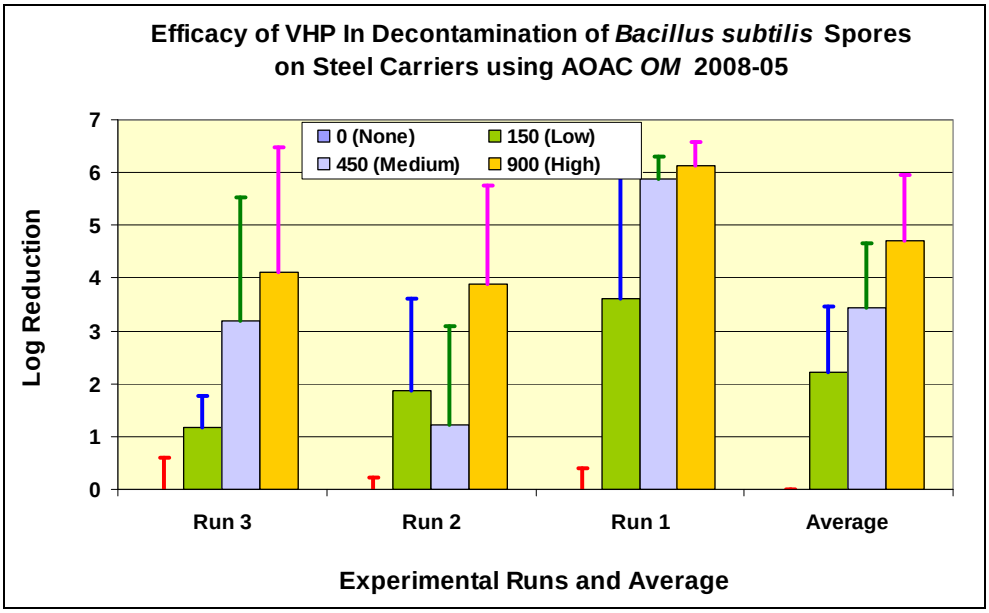

Figure 10. VHP decontamination efficacy of *B. subtilis* spores on steel carriers using AOAC Method 2008-05.

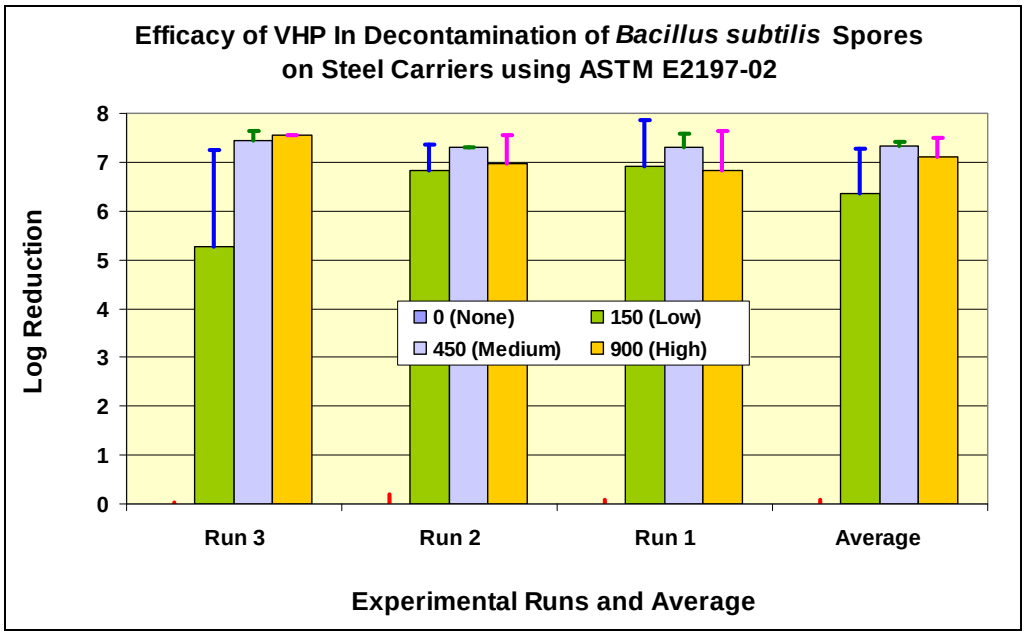

Figure 11. VHP decontamination efficacy of *B. subtilis* spores on steel carriers using ASTM Method E2197-02.

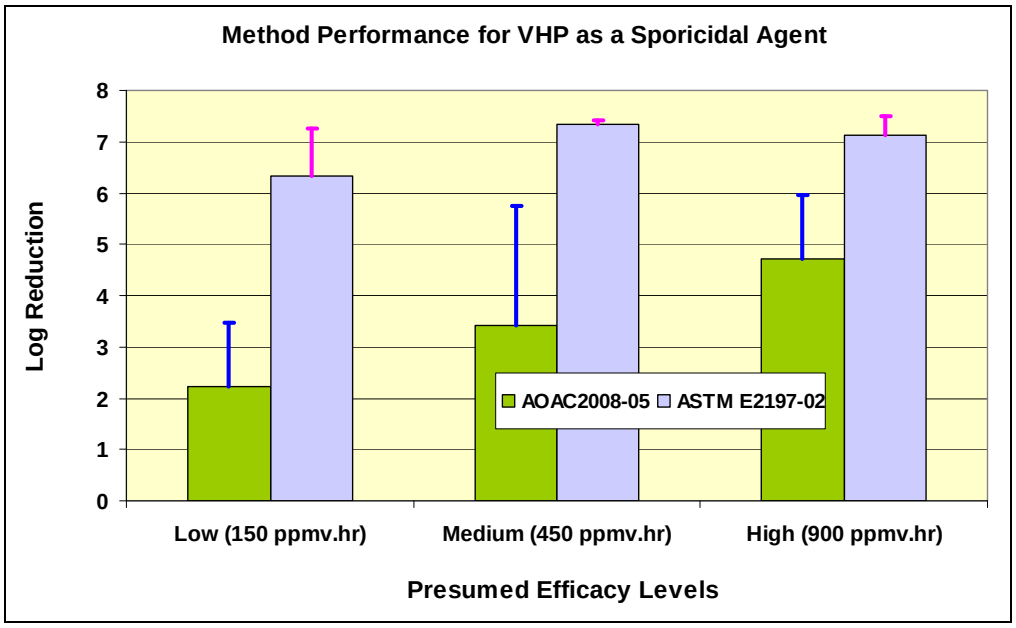

Figure 12. Side-by-side comparison of LR values of both methods after VHP fumigation.

Table 2. LR Summary of Both Methods

Efficacy Level	AOAC Method 2008-05[1]		ASTM Method E2197-02[1]	
	VHP[2]	CD[3]	VHP[2]	CD[3]
Control	0.00 ± 0.19	0.00 ± 0.08	0.00 ± 0.08	0.00 ± 0.06
Low	2.22 ± 1.25	3.76 ± 2.23	6.34 ± 0.92	2.91 ± 0.75
Medium	3.43 ± 2.33	5.02 ± 0.61	7.34 ± 0.08	4.80 ± 1.53
High	4.72 ± 1.24	6.19 ± 0.45	7.12 ± 0.38	6.68 ± 0.48

[1]Average from nine carriers (three per experimental run).
[2]VHP with low, medium, and high CT values corresponded to CT values of 150, 450, and 900 ppmv/h, respectively.
[3]CD with low, medium, and high CT values corresponded to CT values of 250, 500, and 3000 ppmv/h, respectively.

4. DISCUSSION AND CONCLUSIONS

An overall objective of this study was to compare the two validated quantitative methods for liquid disinfection, ASTM Method E2197-02 and AOAC Method 2008-05, for determining the efficacy of VHP and CD gas. It has been shown that VHP and CD gas effectively disinfected the *Bacillus* species (Rastogi et al., 2009), but a standard method was needed to verify this. For both methods, the average spore recovery ranged between 60 and 85% or >7.1 ± 0.41 SD. Therefore, the two test methods are comparable. Significantly high variability (among three replicates within a run or among three experimental runs) was observed with both methods, especially at the low and medium efficacy levels. Despite the high variability with both methods, a trend between high LR values and efficacy treatments was evident.

For VHP efficacy, the ASTM Method E2197-02 did not appear to discriminate between the three treatment levels because similar LR values (5–7) were observed in the results

for all three levels. On the other hand, the VHP results for the AOAC Method 2008-05 showed noticeable differences in the three efficacy treatment levels. However, for CD efficacy, both methods appeared to discriminate among the three efficacy treatment levels.

The variability shown with gas disinfectants is significantly higher than that with liquid disinfectants for the following reasons:

- the two types of steel material may be differentially interacting with the two sterilants;
- spore inoculation over the small area may result in spore stacking and layering; and
- poor penetration of fumigant through the nonviable upper layer of spores may result in varying degrees of protection to the underlying spores.

Because the fumigant within the chamber was circulated using three fans, it is highly unlikely that carrier placement or position could possibly result in observed variability.

We recommend one of the following scenarios for testing:

- use of an inert, hard, nonporous surface, such as glass, to avoid possible contact of the material with the fumigant;
- inclusion of porous surfaces with the quantitative test methods for evaluating gaseous fumigants; or
- use of more than three (perhaps five) replicates subjected to more than three experimental runs (perhaps five).

Another scenario would be to inoculate the carrier with fewer spores to minimize spore stacking (e.g., distribution of 10^7 spores over five or ten carriers instead of one), as is currently required by the quantitative test protocols. We also recommend the inclusion of neutralizer (0.5 M sodium thiosulfate) during spore extraction from test carriers to minimize off-gassing variability. A “universal” test chamber, with humidity, temperature, and fumigant sensors, which could be easily adapted to testing different fumigants, would be an added bonus.

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ACRONYMS AND ABBREVIATIONS

BSC	biosafety cabinet
BSL	biosafety level
CD	chlorine dioxide
CT	concentration × time
ECBC	U.S. Army Edgewood Chemical Biological Center
LR	log reduction
OPP	Office of Pesticide Programs
ppmv	parts per million volume
QCT	Quantitative Carrier Disk Test
SD	standard deviation
TSM	Three Step Method
USEPA	U.S. Environmental Protection Agency
VHP	vaporous hydrogen peroxide

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